The effect of exogenous enzymes and mechanical treatment on mango purée: Microscopic, mesoscopic, and macroscopic evaluation

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Abstract

This paper addresses to what extent mango purée macroscopic properties (consistency) is related to its particle physical properties such as mesoscopic (particle size) and microscopic (morphology) properties. To manipulate mango purée particle sizes, high pressure homogenization (HPH) was used. Commercial fungal and bacterial (cell wall) polysaccharide degrading enzymes without/with HPH (different sequences) were used to understand the contribution of specific (cell wall) polysaccharides and the relevant parameters to consistency changes. The results reflect that, unlike endo-cellulase, pectin methylesterase (PME) together with endo-polygalacturonase (PG), and α-amylase significantly contributed to consistency decrease. HPH only, although largely changing the particle size, did not change the purée consistency, but the combination of HPH with an enzymatic treatment decreased it substantially. Enzymes, while having minimal effect on particle sizes, induced a type-dependent modification of particles' morphology, showing the accessibility of particle polymers (in addition to the expected serum polymers) to enzymes.

Keywords: Exogenous enzymes, High pressure homogenization, Consistency, Mango purée, Particle properties.

1 Introduction

Mango purée, generally preserved by canning, is one of the most popular mango products and is used for the production of jams, squash and juice (Ahmed, Ramaswamy, & Hiremath, 2005; Manohar, Ramakrishna, & Udayasankar, 1991). To produce mango purée, a mechanical disintegration of the tissue is required, whereby a concentrated suspension is formed consisting of mango-tissue-based particles in a continuous serum phase mainly containing soluble polysaccharide such as pectin, sugars and organic acids (Moelants et al., 2013). Pectin is a plant cell wall hetero-polysaccharide rich in galacturonic acid (GalA), which may be methyl-esterified at C-6, and can be O-acetylated at the O-2 and/or O-3. Next to the predominant pectic component GalA, pectin can be composed of 17 different monosaccharides (Vincken et al., 2003; Voragen, Coenen, Verhoef, & Schols, 2009). Due to the process of disintegration, pectin can be either found in the liquid serum phase (soluble pectin) and/or as part of the cell wall material in the particle phase. The amount of pectin in either of the phases depends on the extent pectin is bound to the cell wall, and the processing history (e.g. blanching) prior to disintegration. The amount of pectin and pectin molecular characteristics of both phases influence the rheological properties of the purée by affecting on the one hand particle interactions and on the other hand serum viscosity (Moelants et al., 2012). For example pectin-rich fruits such as mango, having 0.4-0.5% pectin content (fresh weight), yield high viscous juices that together with the pulp form a jellified mass (Kashyap, Vohra, Chopra, & Tewari, 2001; Varanyanond, Naohara, Wongkrajang, & Manabe, 1999; Yashoda, Prabha, & Tharanathan, 2006). Kansci et al., (2008) ascribed such high viscosity of mango purée to its high pectin content. Manohar et al., (1990) stated that the pectin content of mango purée, produced by a fruit pulper, has a critical effect on viscosity to an extent that a 5.7% reduction in pectin content reduced viscosity up to 50%. As mango fruit contains 85% water, concentration of the mango purée is pursued as this process not only minimizes the cost of packaging for export, but also reduces the expense of further processing at destination. However, due to its high viscosity, mango purée cannot be concentrated beyond 30 to 32 °Brix with existing

evaporators and for now any attempt for concentrating mango purée as such would not be without suffering from extensive detrimental effects on its quality (Singh, Dhuique-Mayer, & Lozano, 2000). Next to the pectin content and molecular properties of the pectic polymers of both phases, mesoscopic (particle size) as well as microscopic characteristics (particle morphology) of the particle phase might influence the macroscopic properties (consistency) of the purée (Moelants et al., 2014). In addition, apart from pectin, other polymers such as starch could contribute to the high viscosity of starch containing fruits including mango (~0.1% starch based on fresh weight) (Yashoda et al., 2006). At gelatinization temperature (55-80 °C, which is far below the common blanching temperature, i.e., 95 °C), starch granules absorb water and swell (Swinkels, 1985). This sudden water uptake leads to a strong increase in the system viscosity, but also to enzymatic vulnerability of the starch (Uhlig & Linsmaier-Bednar, 1998).

To date to concentrate a high viscous purée, two approaches have been suggested in literature. One is concentration of mango purée to higher °Brix using the principle of split-stream processing, i.e., separation of serum from pulp followed by concentration of the serum only, that was successful to some extent (Singh et al., 2000). The second suggested approach is enzymatic liquefaction of mango purée followed by concentration (Bhat, 2000; Singh et al., 2000; Sreenath, Nanjundaswamy, & Sreekantiah, 1987; Sreenath, Sudarshana Krishna, & Santhanam, 1995).

Enzymes are used as a processing aid to simplify intermediate processes such as decreasing the viscosity of fruit pulp and increasing filterability of juices in modern food industry (Grassin & Fauquembergue, 1996; Singh et al., 2000). As enzymes are active at low concentration with a reasonable rate under mild processing conditions (temperature, pH) (Fellows, 2000), studies related to the application of exogenous enzymes in food industry have greatly multiplied in recent years (Bhat, 2000; Schmelter, Wientjes, Vreeker, & Klaffke, 2002; Singh et al., 2000; Sreenath et al., 1995). Currently, a combination of pectinases (pectin lyase, pectin methylesterase, endo- and exo-polygalacturonases, pectin acetylesterase, rhamnogalacturonase, endo- and exo-

arabinases), cellulases (endo glucanases, exoglucanases and cellubiases) and hemicellulases (endo- and exo-xylanases, galactanases, xyloglucanases and mannanases), collectively called macerating enzymes, are used for fruit and vegetables juice extraction and clarification, but also to decrease viscosity of fruit purée and facilitate purée concentration (Bhat, 2000). Apart from the abovementioned cell wall degrading enzymes, α-amylase (non-cell wall degrading enzyme) is used to eliminate the starch haze formation for starch containing fruits such as apple, harvested during the early stage, and to reduce the viscosity of such juices (Bhat, 2000; Fellows, 2000). Using exogenous enzymes, one must take two issues into consideration. Firstly, depending on the type of enzymes used, enzyme-treated pectins would potentially represent different applications (Schmelter et al., 2002). Secondly, as each type of fruit has specific quantities and ratios of pectin, hemicelluloses and cellulose in the cell walls, it is necessary to choose the right enzyme preparation in relation to the fruit composition and the final product targeted (Grassin & Fauquembergue, 1996). Modern biotechnology has made it possible to produce purified pectinolytic enzymes at commercial scale (Heldt-Hansen et al., 1996). Hence new doors open towards an efficient application for a tailor-made combination of such enzymes for maximum optimal performance over the present commercial macerating enzymes for certain matrices (Heldt-Hansen et al., 1996).

To obtain low viscosity mango purée from ripe mango, two aspects need to be considered. On the one hand the pectin content of the serum or particle phase should be influenced. This could be achieved by tailoring pectin solubility influencing pectin molecular weight (depolymerizing enzymes) and degree of methoxylation (demethoxylating enzymes). On the other hand, it is known that in ripe mango next to pectin, starch (~0.1% fresh weight) (Yashoda et al., 2006)and rather significant amounts of cellulose in cell walls are present (Mitcham & McDonald, 1992; Ollé, Lozano, & Brillouet, 1996). So the effect of enzymatic degradation of other polymers in mango purée should also be scrutinized.

129 High pressure homogenization (HPH) that is known to result in particle disintegration, on the one
130 hand could assist the enzymatic treatment by increasing the enzyme substrate contact and on the
131 other hand might result in mechanical solubilization of pectin, changing pectin content in serum
132 and particle phase (Moelants et al., 2012).

133 The use of multicomponent commercial enzymes such as Pectinex ®, Celluclast ® and/or a
134 mixture of the two, to manipulate mango purée rheological properties has already been reported
135 in several studies with the aim of optimizing the incubation conditions or investigating
136 physicochemical changes thereof (Bhattacharya & Rastogi, 1998; Singh et al., 2000; Sreenath et
137 al., 1987; Sreenath et al., 1995). However as fruit purées are complex systems, it would be difficult
138 to relate the results observed from multicomponent enzymes to single polymer characteristics. To
139 the best of our knowledge, no studies were performed on the possibility of utilizing HPH separately
140 and in combination with enzymes for decreasing the mango purée consistency. In previous work
141 (Jamsazzadeh Kermani, Shpigelman, Bernaerts, Van Loey, & Hendrickx, 2015), we showed that
142 for both particle and serum phase of mango purée the chemical composition and characteristics
143 of pectin polymers such as degree of methoxylation, average molecular weight, conformational
144 changes of specific polymers, and possible associations and entanglements between the
145 polymers are extensively influenced upon (combined) enzymatic and mechanical treatment.
146 However to what extent such pectin modifications upon treatments influence the macroscopic
147 property (consistency), in relation to mesoscopic (particle size) as well as microscopic (particle
148 morphology) properties of mango purée are still unknown. This study was carried out to investigate
149 how enzymatic treatments with and without HPH affect the consistency of mango purée in order
150 to better understand the parameters resulting in high consistency and how they can be
151 manipulated. Purified fungal and bacterial commercial enzymes (enzymatic treatment) presented
152 the possibility to evaluate the contribution of each individual enzyme to consistency decrease. To

optimize the process and to increase the understanding of the effect of relevant parameters, different sequences of enzymatic and mechanical treatments were applied.

2 Materials and methods

2.1. Plant material

Ripe mangos (*Mangifera indica* L.) of *Keitt* cultivar (pH= 4.2; Brix= 16.8 % ± 0.04) were purchased in a local shop in Belgium. Mangos were thoroughly washed with demineralized water, dried and peeled with a stainless steel knife.

2.2. Sample preparation

The schematic overview of the sample preparation and experimental set-up is presented in Fig. 1. Mango flesh was sliced into layers of 1 cm thickness and vacuum-packed in polyethylene bags in a single layer. To inactivate the endogenous enzymes in order to prevent enzymatic degradation during storage, thawing, and further manipulation, mango flesh was blanched for 8 min at 95 °C in a temperature-controlled water bath. Immediately after the treatment, the bags were transferred to an ice bath, frozen with liquid nitrogen and stored at -40 °C.

Mango purée from blanched slices was obtained by blending (Waring blender 8010 EB, Torrington, CT, USA) 600 g mango slices for 30 s at high speed. To prevent biased conclusions originating from variability of individual specimens, for the whole experimental set-up 44 kg of blanched purée was prepared likewise and mixed. Aiming different objectives, mango purée was processed using five different treatments, i.e., mechanical treatment (HPH), enzymatic treatment, HPH followed by enzymatic treatment, enzymatic treatment immediately followed by HPH, incubation of purée with enzymes followed by HPH. All treatments were performed in duplicate. All samples were re-blanched (8 min at 95 °C) after the above mentioned treatments to inactivate the exogenous enzymes added. Samples without added enzymes were also blanched aiming for

a fair comparison between enzymatic and non-enzymatic treatments. After blanching, purées were cooled in an ice bath and equilibrated at 25 °C for 15 min. The treated purées were subsequently used for consistency and particle size distribution measurements and microscopic imaging.

2.3. Mechanical treatment

Mango purée was first defrosted in a water bath at 25 °C for approximately 15 min. Then demineralized water was added (50% of the total amount of aqueous phase added at the end, 10 ml for 200 g of purée), and the pH was adjusted to 5.0. The obtained purée was homogenized at 20, 60 or 100 MPa via a single pass, using a Panda 2k high-pressure homogenizer (Niro Soavi, Parma, Italy), of which the in- and outlet were thermostated at 4 °C using a cryostat (Haake, Karlsruhe, Germany). Homogenized purées were vacuum packed in plastic bags and incubated for 1 h in a shaking water bath at 40 °C. Immediately after incubation, the bags were put in a water bath at 95 °C for 8 min and cooled in an ice bath for 5 min. Taking into account the volumes of water and alkaline solution (1M NaOH) already added in the previous steps to adjust pH, demineralized water was added to obtain purée with a ratio of 1:10 added aqueous phase:purée (at the end, having 20 ml of aqueous phase for 200 g of purée).

2.4. Enzymatic treatment

Commercial pectinmethylesterase (PME) (Novoshape, Novozymes), and ultrapure endo-polygalacturonase (PG) from *Aspergillus aculeatus* (Megazyme), bacterial α-amylase from *Bacillus sp.* (Sigma-Aldrich) and fungal endo-cellulase (endo-β-glucanase) referred as cellulase in this paper from *Aspergillus niger* (Megazyme) were used. Preliminary experiments showed complete solidification of mango purée using PME alone (results not shown), suggesting that possible impurities can be considered negligible. CBM3a, an antibody to recognize crystalline cellulose, did not label the control and cellulase treated samples (images not shown). It might be

speculated that if present, the crystalline cellulose part was sterically inaccessible for the antibody. This was one of the reasons why pure endo glucanase was used for the enzymatic treatment as this enzyme specifically degrades amorphous cellulose (Cao & Tan, 2002).

After defrosting the purée, pH was adjusted to the optimal pH as claimed by the provider (5.5, 6.0. 4.5, 5.5) for each of the enzymatic treatments: “PG and PME”, “α-amylase”, “cellulase”, and “combination of PG and PME, α-amylase and cellulase” referred to as “all enzymes” treated purée, respectively. One unit[1] (U) of each individual enzyme was added to 1 g of total purée (including the added aqueous phase) (Table 1). All purées were vacuum packed in plastic bags and incubated under the same conditions (shaking 1 h at 40 °C). Thermal treatment (8 min at 95 °C) was used to inactivate the enzymes after incubation. The pH of the purées was re-adjusted to 5.0. Finally, demineralized water was added to obtain the ratio of 1:10 aqueous phase:purée considering the volumes of added water, enzymes, alkaline (1M NaOH) and acid (0.1M HCl) solutions (used for pH adjustments) (Jamsazzadeh Kermani et al., 2015).

2.5. Combination of mechanical and enzymatic treatment

2.5.1. High pressure homogenization followed by enzymatic treatment

Mango purée was initially homogenized at 20 MPa using a Panda 2k high-pressure homogenizer (Niro Soavi, Parma, Italy), followed by pH adjustment (to the optimal pH of the applied enzymes) and enzyme addition. Compared to the enzymatic treatment only, the combined effect of HPH and “PG and PME” together with “α-amylase” on the properties of the purée obtained was also evaluated. For such treatment, the pH of the mango purée was adjusted to 5.5 prior to addition of

[1] One unit of PG is defined as the amount of enzyme releasing 1 µmol of galacturonic acid per min at pH 5.5 at 40°C (Combo, Aguedo, Goffin, Wathelet, & Paquot, 2012). One unit of PME is defined as the amount of enzyme catalyzing the hydrolysis of 1 µmol of ester bonds per min at pH 7.0 and 22 °C. One unit of α-amylase liberates 1.0 mg of maltose from starch in 3 min at pH 6.9 at 20°C (Enzymatic assay, Sigma).One unit of cellulase is defined as the amount of enzyme required to release 1 µmol of 2-*chloro*-4-nitrophenol from CellG3 in 1 min under assay conditions at 40 °C (Enzymatic, Megazyme).

the enzymes. The same proportion of enzymes, incubation and blanching conditions were used as in the enzymatic treatment described in Section 2.4. The pH was re-adjusted to 5.0 and water was added to achieve the ratio of 1:10 aqueous phase:purée, analogous to the enzymatic treatment. For the control purée the addition of exogenous enzymes was excluded (Jamsazzadeh Kermani et al., 2015).

2.5.2. Enzymatic treatment immediately followed by HPH

For this treatment, after the addition of respective enzymes (Section 2.4), purées were immediately homogenized at 20 MPa and the homogenized purées were vacuum packed in plastic bags and incubated for 1h at 40 °C in a shaking water bath. After incubation, the pH was re-adjusted to 5.0 and the final ratio of aqueous phase:purée was adjusted to 1:10. For the control purée, the addition of exogenous enzymes was excluded.

2.5.3. Incubation of purée with enzymes followed by HPH

Similar to Section 2.5.2, exogenous enzymes were added to purées but the HPH treatment was performed after incubating the purées for 1h at 40 °C. Before the HPH treatment, the pH of the purées was adjusted to 5.0 and purées subsequently homogenized at 20 MPa. The ratio of aqueous phase to purée was adjusted after HPH. For the control purée, the addition of exogenous enzymes was excluded.

2.6. Rheological characteristic of mango purée

Bostwick consistency measurement

In practice, Bostwick consistency is frequently used to describe the rheological behavior of a non-Newtonian food product such as fruit purée (Perona, 2005). In this emperical test, the sample flows under its own weight at certain temperatrue for 30 s. The distance that the sample flows over this time is measured and reported as Bostwick consistency index (Christiaens et al., 2012). Bostwick consistency summarize variability of viscosity into a unique mean value and quantifies

such value as the mean resistance that the fluid experiences against deformation under gravity-driven flow. Since this value could be an equivalent for the mean viscosity, this technique is of large inteterst thanks to its speed and simplicity (Perona, 2005). A high Bostwick consistency index corresponds to a purée having low consistency which has a low resistance to flow and vice versa. Approximately 100 g mango homogenate (24 °C ± 1) was poured into the reservoir and after an equlibration time of 1 min in the reservoir, the consistency was assessed by opening the gate of the reservoir of Bostwick consistometer (Tibäck et al., 2009). All measurements were conducted in duplo.

2.7. Structural characteristic of the particles

Particle size distribution

The particle size distribution (PSD) of mango purée was measured using laser diffraction (Beckman Coulter Inc., LS 13 320, Miami, Florida). Hereto, samples were poured into a stirred tank, filled with demineralized water and pumped into the measuring cell wherein the laser light (wavelength main illumination source: 750 nm; wavelengths tungsten-halogen light for Polarization Intensity Differential Scattering (PIDS): 450 nm, 600 nm, 900 nm) was scattered by the particles (particles between 0.04 µm and 2000 µm can be detected) (Verrijssen et al., 2014). The volumetric PSDs were calculated from the intensity profile of the scattered light with Fraunhofer optical model using the instrument`s software. Some deviations from the correct particle size might occur for the samples where most of the particles are <10 µm due to possible deviation from the Mie theory (Jamsazzadeh Kermani, Shpigelman, Pham, Van Loey, & Hendrickx, 2015).

2.8. Bright field microscopy

Microscopic pictures were taken to visualize the microstructure of the particles after enzymatic and mechanical treatment. Light microscope (Olympus BX-41) equipped with an Olympus XC-50

digital camera (Olympus, Opticel Co. Ltd., Tokyo, Japan) was used with an objective of 10× or 40× for magnification using either bright field or differential interference contrast mode (DIC). Iodine solution (containing 0.2% iodine and 2% potassium iodide) and toluidine blue solution were used to stain starch polymers and the cell walls respectively. At least ten images were obtained per sample.

2.9. Statistical analysis

Differences in mean values for the Bostwick consistency indices were estimated using one-way ANOVA. Pairwise comparison of the means were performed using the Tukey's Test ($P<0.05$) (Origin Pro, version 8.0724, MA01060 U.S.A.).

3 Results and discussion

The different treatments used in this study aimed at different goals. The objective of the HPH treatment only, was mainly to affect the particle sizes by disaggregation and disruption of cell clusters and individual cells in the purée and investigate to what extent such changes influence Bostwick consistency of the purée. Enzymatic treatments were performed with the aim of mainly influencing enzyme-accessible polymers from serum and particle phase. In our previous study (Jamsazzadeh Kermani et al., 2015), it was shown that when (non) homogenised purée was enzymatically treated, the chemical properties of pectin polymers of the serum as well as the paricles were largely influenced. Therefore to understand whether there is a mutual synergistic effect of HPH and enzymatic treatments on the flow properties of mango purée, such treatments were applied in differenct orders. Hereto, when HPH followed by enzymatic treatment was performed, the goal was to generate more accessible substrates for the enzymes by cells disruption while the aim of enzymatic treatment immediately followed by HPH was to investigate whether possible stimulation of enzymes due to HPH can modify polysaccharide structure and affect consistency. Finally, when purées were enzymatically treated for 1 h and subsequently

292 homogenised, the goal was to investigate the sensitivity of the enzyamtically modified systems to
293 mechanical treatment thereof.

294 ### *3.1. State of starch prior to enzymatic treatment*

295 Starch gelatinization is a prerequisite for enzymatic hydrolysis of starch by α-amylases. To confirm
296 that the applied blanching condition (95 °C for 8 min) prior to enzymatic treatment gelatinized
297 mango starch, the effect of the blanching on the starch granules was visualized using polarized
298 light and is shown in Fig. 2. It is clear from Fig. 2 that when blanched mango purée (Fig. 2B) and
299 mango flesh tissue (Fig. 2A) were compared under polarized light, no Maltese crosses (loss of
300 birefringence) for the blanched purée were observed even for the smallest starch granules which
301 are believed to be gelatinized at higher temperature (Swinkels, 1985). This shows that the applied
302 blanching temperature was beyond the temperature range of mango starch gelatinization and all
303 the starch granules were in the gelatinized state prior to enzymatic treatment. This is in agreement
304 with previous published data on mango starch gelatinization for which a gelatinization temperature
305 of 73.8 °C was reported (Millan-Testa, Mendez-Montealvo, Ottenhof, Farhat, & Bello-Pérez,
306 2005). On the other hand, when mango flesh tissue and blanched mango purée were stained by
307 iodine solution and visualized using differential interference contrast mode (DIC) (Fig. 2A and 2B),
308 giant starch granules in a purplish background was observed. As appreciable swelling, and
309 noticeable disruption of the starch granules (observed as swollen granule fragments (Fig. 2B))
310 occurred, it seems that apart from the gelatinization temperature and loss of birefringence, the
311 pasting temperature was reached. Reaching of mango starch to pasting temperature upon
312 blanching prior to enzymatic/combined enzymatic and mechanical treatment might explain a
313 partial potential role of starch to the high consistency of the blanched mango purée (Swinkels,
314 1985).

3.2. Effect of mechanical and enzymatic treatment on the consistency of mango purée

The Bostwick consistency (BC) indices for all (non-) mechanical and/or enzymatic treated mango purée is shown in Fig. 3A-E. For mechanical treatment only (Fig. 3A), non homogenized purée was considered as the control sample and compared with purée homogenised at 20, 60, and 100 MPa. The BC of non homogenised mango purée was 6.6 cm. Statistical analysis showed no significant difference between the BC indices of homogenised mango purées including the control sample.

When enzymatic treatment only was applied, except for the cellulase treated purée, BC indices (Fig. 3B) indicated a clear significant improvement (reduced consistency) compared to the non enzymatically treated purée. It is obvious that applying cellulase, had no effect on BC compared to the control. However when pectin polymers were demethoxylated and depolymerised by PME and PG, less resistence to flow was observed. Depolymerization of starch by α-amylase also decreased the consistency (9.9 cm) compared to the control purée (6.6 cm). The combination of all enzymes resulted in a two-fold decrease in BC index of mango purée compared to non enzymaticaly treated purée and a significant decrease was also observed compared to the use of "PG and PME" only. However by using the mixture of all enzymes and degrading starch on the one hand and demethoxylating and depolymerising pectin on the other hand, no additional effect on decreasing consistency was observed compared to "PG and PME" and "α-amylase" only.

When the combination of the HPH and enzymatic treatment was used, the pressure level of 20 MPa was selected because of two practical reasons. On the one hand and based on the results from the mechanical treatment only, no significant effect on the BC indices were observed as the pressure level further increased. On the other hand homogenization of a very viscous non diluted purée at higher pressure levels was practically inconvenient.

339 The BC indices for the purée which was first homogenised (20 MPa) and then enzymatically
340 treated are reported in Fig. 3C. The BC indices of homogenized and enzymatically treated purée
341 showed a similar trend in consistency reduction as the enzymatic treatment only. However the
342 effect of “all enzymes” after homogenization (12.8 ± 0.4 cm) on the consistency decrease was
343 significantaly ($p<0.05$) larger than the effect of “all enzymes” only (10.6 ± 0.2 cm). Additionally,
344 unlike the enzymatic treatment only (Fig. 3B), when enzymes were applied on the homogenised
345 purée (Fig. 3C) a significant difference in BC indices was observed for “all enzymes” compared
346 to “PG and PME” and “α-amylase” treated purées.

347 If HPH is applied immediately after the addition of enzymes a significant but a small increase was
348 observed for BC indices of purée treated with “α-amylase”, “PG and PME”, and a combination of
349 all enzymes compared to HPH treated purée (control of this set of treatment) (Fig. 3D).
350 Comparison of the Fig. 3B, 3C, and 3D showed that when mechanical treatment was combined
351 with enzymatic treatment, the order of these two treatments plays a crucial role in determining the
352 mango purée consistency especially in case of “all enzymes”. While enzymatic treatment of the
353 HPH treated purée significantly increased the consistency index (12.8 ± 0.4 cm) (Fig. 3C)
354 compared to enzymatic treatment only (10.6 ± 0.2 cm) (Fig. 3B), the consistency index showed a
355 significant decrease when HPH was applied immediately after the addition of enzymes (7.4 ± 0.4
356 cm) (Fig. 3D). This is possibly due to the enzymatic activity during a short inevitable holding time
357 before the start of the homogenisation. After HPH, however, the rearrangement of the polymers
358 due to the breakage of the particles induced by HPH treatment might decrease the substrate
359 accessibility for the enzymes. This might be the reason for the small furthur improvement of BC
360 indices observed, after the incubation time compared to control (Fig. 3D).

361 When HPH treatment was applied on enzymatically treated and incubated purée, no significant
362 increase of BC indices was observed (Fig. 3E). From Fig. 3B, it was concluded that the enzyamitc
363 treatement only, reduces the consistancy, so it is clear that the HPH treatment of enzymatically

treated purées resulted in an opposite effect. It seems that after the degradation of the polymers by enzymes, some polymer and particle rearrangements were induced by homogenization, resutling in an unexpected increase of consistency (Jamsazzadeh Kermani et al., 2015). It can be hypotheised that such rearrangements might lead to the formation of a structured network and subsequently increase the purée resistance to flow. However the mechanism of how homogenisation of enzyamtically treated purée induced the consistency increase is not clear and requires further research.

3.3. Effect of mechanical and enzymatic treatment on the particle size of mango purée

The volumetric PSD for all (non-) mechanical and/or enzymatic treated mango purée is presented in Fig. 4A-E.

As shown in Fig. 4A, a bimodal particle size distribution is observed for the non homogenised purée, with maximum peaks around 100-200 µm and 600-700 µm particle diameter. As it was expected, HPH clearly decreased mango purée particle sizes. By increasing the homogenization pressure up to 100 MPa, particle size reduced to almost 10 times smaller compared to the particles of non homogenised purée. These small particles resulted from breaking of the large particles, led to a purée having more homogenous particle size distribution. As no significant differences were observed between BC indices of HPH treated purées compared to the non-homogenised one, it is striking that the particle size was not such an important factor in determining the consistency of mango purée. However, one should keep in mind the importance of both phases (particle and serum) to the final consistency observed. It was previously reported that a decrease in particle size reduces the viscosity for systems such as apple purée (Espinosa et al., 2011), hence it is possible that the decrease in particle size, reduced the consistency, but on the other hand an increase in polymers concentration in serum due to the release of the soluble cell content

388 simultaneously increased the consistency and masked such effect (Jamsazzadeh Kermani et al.,
389 2015).

390 Comparison of the PSDs for enzymatically treated purées (Fig. 4B) showed that for all treated
391 purées a bimodal PSD profile with similar average sizes as for non enzymatic treated purée was
392 present. However, the ratios between the peaks in the bimodal distribution changed. Such purées
393 also showed an increase in BC indices (Fig. 3B). While the purée treated with cellulase (with no
394 significantly different BC compared to control) had a similar ratio for both peaks compared to the
395 non enzymatic treated purée, for purées treated with α-amylase and all enzymes a visible change
396 in ratio of the peaks around 100-200 µm and 600-700 µm compared to the non enzymatic treated
397 purée was observed. A decrease in the volume percentage of particles with diameter around 600-
398 700 µm resulted in volume based increase in particles with diameter around 100-200 µm.
399 Moreover, the combination of all enzymes also led to a significant increase in the volume occupied
400 by small particles with sizes around 1-20 µm. As α-amylase was the common enzyme present in
401 both these treated purées, it can be suggested that the observed decrease in volume percentage
402 of the particles with diameter around 600-700 µm compared to non-treated, “cellulase”, and “PG
403 and PME” treated purée was partially due to the starch degradation by α-amylase. While for “PG
404 and PME” treated purée no specific changes on the PSD profile was observed, there was a
405 significant difference in the BC index compared to the control purée. This observation
406 strengthened our previous conclusion based on PSD profile (Fig. 4A) and BC indices (Fig. 3A) of
407 HPH treated purée, that for mango purée the particle size is not the major factor responsible for
408 consistency differences observed.

409 Fig. 4C represents the volumetric PSD of the purées, which are initially homogenised and
410 subsequently treated with enzymes. It is noteworthy that when homogenised purée was treated
411 with enzymes, changes in the volumetric PSD profile due to the enzymatic treatment were limited
412 to “all enzymes” and “PG and PME” treated purées, in which the PSD profile showed more

homogenity, having a sharper main peak. The presence of PG and PME resulted in a small increase in particles with a diameter around 1-20 µm. By and large, use of HPH before using a combination of all enzymes, did not significantly change the PSD profile, however a significant decrease in BC index was observed for HPH purée treated with combination of all enzymes.

By changing the sequence of the treatment being first an enzymatic treatment immediately followed by HPH (Fig. 4D), different PSD profiles were observed for “cellulase”, “PG and PME”, “all enzyme” purées compared to the control purée. Althought it was previously stated that enzymes alone did not induce a dramatic influence on the PSD profile, particles treated with different enzymes did not respond similarly to the subsequent HPH treatment as is infered from Fig. 4D. The short holding time between the addition of enzymes and HPH treatment seemed to be sufficient for enzymes to sensitise particles to the mechanical treatment especially for purées treated with PG and PME, and the combination of enzymes resulting in the formation of smaller particles due to HPH compared to control purée (Fig. 4D).

The volumetric PSD of the purées that were enzymatically treated for 1 h and consequently homogenised is shown in Fig. 4E. An increase in the volume occupied by small particles (0.1-10 µm) was observed after homogenisation of purée incubated with “PG and PME” and “all enzymes” compared to only homogenised purée (control of this set of treatment). Generally, the vast numbers of small particles occupied the same volume for which far less numbers of large particles are required. It is striking that pectin degrading enzymes had a clear impact on the cell wall polymers present in particle phase in such a way (Jamsazzadeh Kermani et al., 2015) that after homogenisation, significant numbers of particles with more than 10 times smaller size were produced compared to only HPH treated purée. It seems that the appearence of these small particles resulted in the suggested rearrangement of polymers leading to the high consistency of purée (Fig. 3E) obtained with such an order of treatments.

3.4. Structural characteristics of particles: particle type

The average diameter of a mango tissue cell is between 80 to 150 µm (Joyce, Shorter, & Hockings, 2001). Consequently, based on the PSD results, the non homogenised non enzymatic treated purée consisted mainly of clusters and partially particles with the size of individaul cells. Increasing the intensity of the high pressure homogenisation treatment increased the volume percentage of the smaller particles. As the measurement of particle sizes by light scattering technique is based on spherical particles, and the tissue based particles of fruit suspensions are anisotropic in shape (Lopez-Sanchez et al., 2011), from the PSD results only, no conclusion can be made regarding particle morphology. Hence it is not clear whether those small particles are individaul cells or anisotropic remnants of neighboring cells. The knowledge of particle morphology helps us to better describe whether the decrease in particle sizes was mainly the result of cell breakage or cell separation. Depending on the mechanism behind the particle size reduction (cell breakage or cell seperation), the composition of the serum largely differs. Cell breakage is believed not only to increase particle numbers (concentration), but also to induce cell content release and to increase the serum polymer (particularly starch) concentration, while cell separation increases particle numbers as well as the possible pectin polymer concentration of the serum by partial solubilisation of pectin from middle lamella (Jamsazzadeh Kermani et al., 2015).

Microscopic images were used not only to confirm the size, but more importantly the morphology of the particles. On the micrographs of Fig. 5, purée particles obtained after HPH at 20, 60, 100 MPa were stained with toluidine blue (Fig. 5A) and iodine solution (Fig. 5B) to generally stain the cell wall material and starch polymers, respectively. Increasing the intensity of the HPH treatment led to drastic changes in the morphology of the mango purée particles. It is clear from Fig. 5A that the particles had “rough edges” (Lopez-Sanchez, Chapara, Schumm, & Farr, 2012) not only for the control but also for all homogenised purées ranging from compact clusters (20 MPa, 60 MPa) to broken cells (60 MPa, 100 MPa). Microscopic images (Fig. 5A) showed that the decrease in

462 particle size by intensive mechanical treatment was mainly due to the cell breakage. The pH of
463 the mango purée during the first blanching step (95 °C for 8 min) was 4.2 ± 0.1, and β-elimination
464 reactoin (a chemical depolymerisation reaction of pectin at T>80 °C and low acid and alkaline pH)
465 (Sila et al., 2009) might partially degrade pectin under such conditions. However particles
466 measured as the size of mango cells (Fig. 4A), were hardly individual cells in any of the (non)
467 homogenised samples (Fig. 5A). One can conclude that the middle lamella in mango fruit
468 parenchymatous tissue was not heat labile, and the heat load in the first blanching step and/or the
469 pH did not result in pectin degradation in this layer to make the mango tissue and purée particles
470 consisting of cell clusters, susceptible to cell separation by blending or HPH, respectively. Hence
471 during the two mechanical steps of purée preparation, i.e. blending and HPH (intensive disruption),
472 cells were broken across the cells, resulting in formation of particles with rather more anisotropic
473 shapes with rough surfaces. On the one hand the cell breakage released the gelatanised starch,
474 which was previously located inside the cells, to the serum phase, increasing the concentration of
475 this phase. On the other hand it induces the formation of many sharp edges on particle surfaces.
476 Although the size of the particles became smaller due to HPH and hence less resistence to flow
477 was expected, it can be suggestted that the formed sharp edges increased the friction and/or
478 induced better association of the particles increasing the resistence to flow. Or it could be that by
479 breaking cells starch granules were released into the serum phase and increase the consistency.
480 These two counteracting effects, resulted in in lack of consistency change due to HPH.

481 As seen in Fig. 5B, HPH also decreased the size of the gelatinised starch granules, that have
482 been released from the cells to the serum phase.

483 Modification of the cell wall material by enzymatic treatment was also visualised with toluidine blue
484 and is presented in Fig. 6A. Clear differences in particle morphology were observed using different
485 enzymatic treatments. For the control purée, not only the sharp edges were observed on the
486 surface of the clusters, but also stained closed circles spreading over each of the cells (inside of

the cluster). As stated before, sharp edges were an indication for cell breakage, however the stained closed circles on the cell surfaces indicated that cell separation also occurred on milieu of such circles supposed to connect different cells. It seems that the cell separation was selective for junctions zones, and the cell breakage was selective for middle lamella. When purée was treated with cellulase, the morphology of the particles did not change. However the cell wall material was less densely stained due to the digestion of cellulose. For “α-amylase” treated purée, the cell wall seemed to be thicker compared to the control. Perhaps degradation of the free gelatinised starch in the serum phase by α-amylase, freed and mobilised the water in the system which was previousely captured by starch during gelatinization. This phenomenon helped part of the pectin of the particle phase to absorb that freed water inducing thicker cell wall observed. For “PG and PME” treated purée and when the combination of all enzymes used, significant changes in the morphology to complete disruption of the particles, respectively were observed. This shows that the enzymatic degradation of pectic polymers is not only limited to soluble polymers in the serum phase but also pectic polymers of the particles that are playing a cruicial role in cell wall integrity.

The microscopic images of non-/enzymatically treated mango purée stained with iodine solution are also presented in Fig. 6B. The size of the starch granules in mango was reported to be 10 μm (Bello-Pérez, paricio-Saguilán, Méndez-Montealvo, Solorza-Feria, & Flores-Huicochea, 2005), so the observed giant stained areas (Fig. 6B) are the granules that continue to expand by hydration of amorphous region to a greatly swollen reticulated mesh like network, but still held together by persistent micelles via hydrogen bonds in the non-disrupted possible crystelline regions (Swinkels, 1985). The swollen starch (dark purple), still located inside the cells, can be clearly observed for the control purée and purées treated with cellulase and the combination of PG and PME. It is interesting that for “PG and PME” treated purée, in which the cell walls became disintegrated (Fig. 6A), less intense staining was observed. This might be ascribed to the increased mobility of the water towards the starch granules through the cell wall after the enzymatic treatment, leading to

512 increase in swelling of the starch in the final blanching step and hence less intense staining with
513 iodine solution. The presence of swollen granules in non α-amylase treated purée indicated that
514 gelatinised starch are still integrated even after the high temperature blanching and the blending
515 shear used. Although the total amount of starch in ripe mango is reported to be limited, it should
516 be considered that even such little amounts, if not hydrolysed, could make a composite gel
517 network of swollen amylopectin-enriched granules in an interpenetrating amylose gel matrix
518 (retrogradation) after the subsequent cooling and during storage (bd Karim, Norziah, & Seow,
519 2000; Miles, Morris, Orford, & Ring, 1985). This process seems to contribute to consistency
520 increase in the final product (bd Karim et al., 2000), indicating the importance of selecting proper
521 enzymes based on the biological source. For purées treated with α-amylase and all enzymes, no
522 stained starch could be found. One must take this into consideration that staining of starch with
523 iodine solution is based on the formation of an amylose-iodine complex. When amylose is present
524 in the form of a helix, a core of iodine molecules can be formed inside the helical structure. The
525 formed complex is responsible for the blueish color (Gilbert & Marriott, 1948). However, the blueish
526 color is only obtained when the amylose chain consists of not less than 45 glucose units (Wilding,
527 1965). The absence of iodine solution staining for the purées treated with α-amylase and all
528 enzymes does not necessarily mean that no starch polymers were present, but it shows that starch
529 might still be present probably not in the form of helices (< of 6 glucose units) and/or that the chain
530 length of the remaining amylose helices is less than 45 glucose units to form the color. Lack of
531 starch staining even when “PG and PME” were not used, showed that all starch in mango purée
532 was accessible by α-amylase.

533 ### *3.5. Relation between Bostwick consistency and parameters influenced by*
534 ### *enzymatic and HPH treatment*

535 To identify the main parameters influencing mango purée consistency, a correlation plot between
536 different parameters of the serum and particle phase in this paper and previous work

(Jamsazzadeh Kermani et al., 2015) as influenced by the enzymatic treatments and the combination of HPH and enzymatic treatment is shown in Fig. 7. In general, the amount of serum is the only parameter that had a strong and positive correlation with BC index, meaning that release of water, most likely with some degraded polymers into serum, increased the BC index (decrease consistency). On the other hand no correlation was observed between BC index and the amount of estimated pectin of serum indicating that soluble pectin was not the main polymer defining the consistency of the mango purée. Rather strong negative correlation was observed between BC index and DM of the serum. As together with PME, PG was also always used and as low methoxylated pectin is a preferred substrate for PG, it can be concluded that low DM was corresponding with relatively lower M_w of pectin polymers due to the break down of pectin HG backbone. Such polymers however were supposed to be extremely branched as no side chain degrading enzymes was used for such treatments. However this hypothesis is not in full agreement with rather strong negative correlation between the DM and M_w of polymers of the serum. The reason for this negative correlation is that M_w was calculated for all serum polymers assuming the same dn/dc such as starch and pectin. The lack of correlation between the BC index and amount of estimated serum pectin is not in agreement with previous data observed for the tomato sera by Moelants et al. (2012). The aouthors showed that the amount of pectin affected the viscosity of the tomato sera. This might be explained by different polymeric composition of tomato and mango suspentions for which starch showed to be one of the main contributing factors for purée consistency.

The negative correlation between the BC index and the DM of pectic polymers was stronger than the correlation of the consistency index and the M_w of the serum, which was another evidence that pectin was not the only polymer contributing to the consistency. One must take into consideration that although the polymers of particle phase and particularly pectin polymers were largely influenced by the enzymatic and the combination of HPH and enzymatic treatment, such water

562 solublised polymers were still part of non soluble particle phase, and did not or even negatively
563 correlate with the consistency of the mango purée.

564 As is clear from the correlation plot, particle sizes are strongly and negatively correlated with both
565 the amount of serum and the BC index. However from our results the mechanism behind this
566 correlataion is not completely clear.

567 *4 Conclusion*

568 In this research, it was observed that the high consistency of mango purée can not be solely
569 related to pectin polymers and their modifications. Other polymers such as starch and cellulose
570 are also contributing to the high consistency observed whether directly (starch) or indirectly
571 (cellulose). It was also observed that the enzymatic treatment significantly changed particle
572 morphologies. Our results suggest that particle morphology is an important factor in consistency
573 changes in mango purée. The sequence by which the enzymatic and mechanical treatments is
574 applied are crucial to the final consistency observed. When the mechanical treatment is followed
575 by the enzymatic treatment, the largest decrease in consistency was observed probably due to
576 the higher accessibility of the enzymes to the polymeric material. Based on the comparison
577 between the BC indices and microscopic images of homogenised and non-homogenised mango
578 purée, it was concluded that the particles size and release of cell content due to the cell breakage
579 was not a determining factor for mango purée consistency. When the purée was first homogenised
580 and then a combination of enzymes (pectin methyesterase, endo polygalacturonase, α-amylase
581 and endo-cellulase) was used the BC index changed by a factor of two. In general, the amount of
582 serum is the main parameter showing a strong and positive correlation with BC index, meaning
583 that release of water and most likely with some degraded polymers into the serum by the
584 enzymatic treatments increased the BC index (decreased the consistency).
585 While our conclusions may be relvent to many other fruit and vegtable purées, one must take into
586 consideration that direct extrapolation of these results is not advisable without appropriate testing

587 due to the large variation of pectin structure and other components within spieces. The results of
588 this study lift the tip of the veil of how the selection of enzymatic treatment, and the sequence of
589 mechanical and enzymatic treatment influence the consistency of purées. No doubt that future
590 reseaerch would benefit from focusing on the relation between the modified structure of starch
591 and cell wall polysaccharides and their observed functionality due the enzymatic and HPH
592 treament. A subject which received little attention in literature related to mango purée processing.
593 Additionally, it would be worthwhile that future research is directed towards the analysis of the
594 sensorial properties of the enzymaically treated samples, as for example the enzymatic conversion
595 of the strach in “α-amylase” treated samples might result in a sweeter product without using any
596 added sugar.

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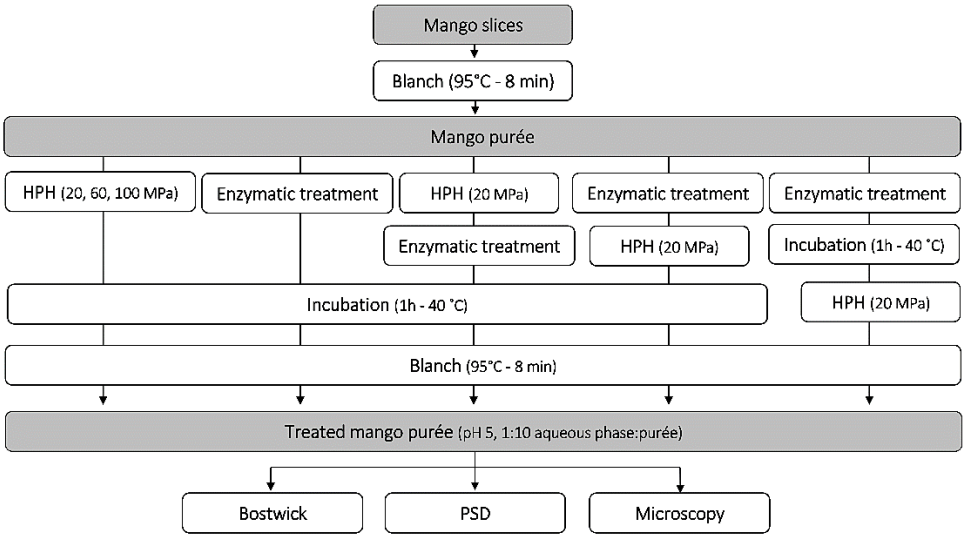

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Fig. 1 Schematic overview of the experimental set-up. HPH, high pressure homogenization; PSD, particle size distribution. Enzymatic treatments of purée were treatments with “PME+PG”, “α-amylase”, “cellulase” alone and “combination of PME, PG, α-amylase, and cellulase”.

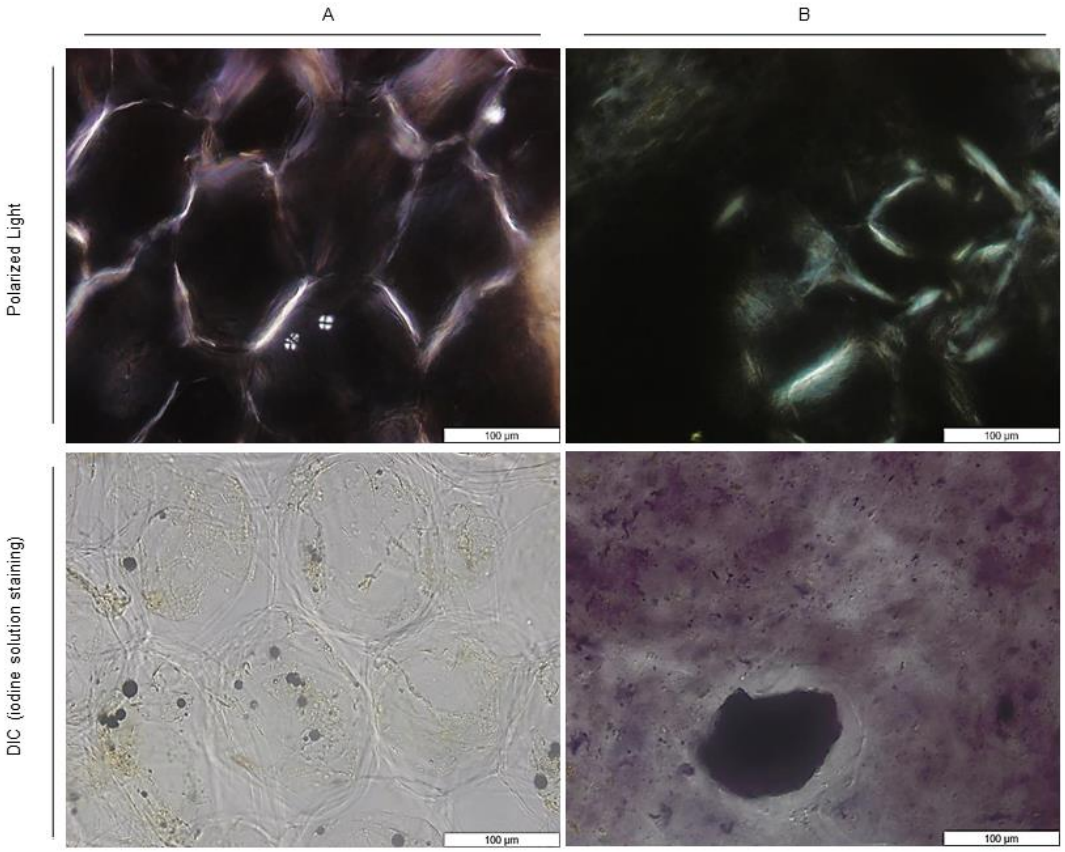


Fig. 2 Microscopic images of (A) mango flesh tissue, and (B) blanched mango purée under polarized light (upper row), and with iodine solution staining under differential interference contrast mode (DIC) (lower row). Scale bars = 100 µm.

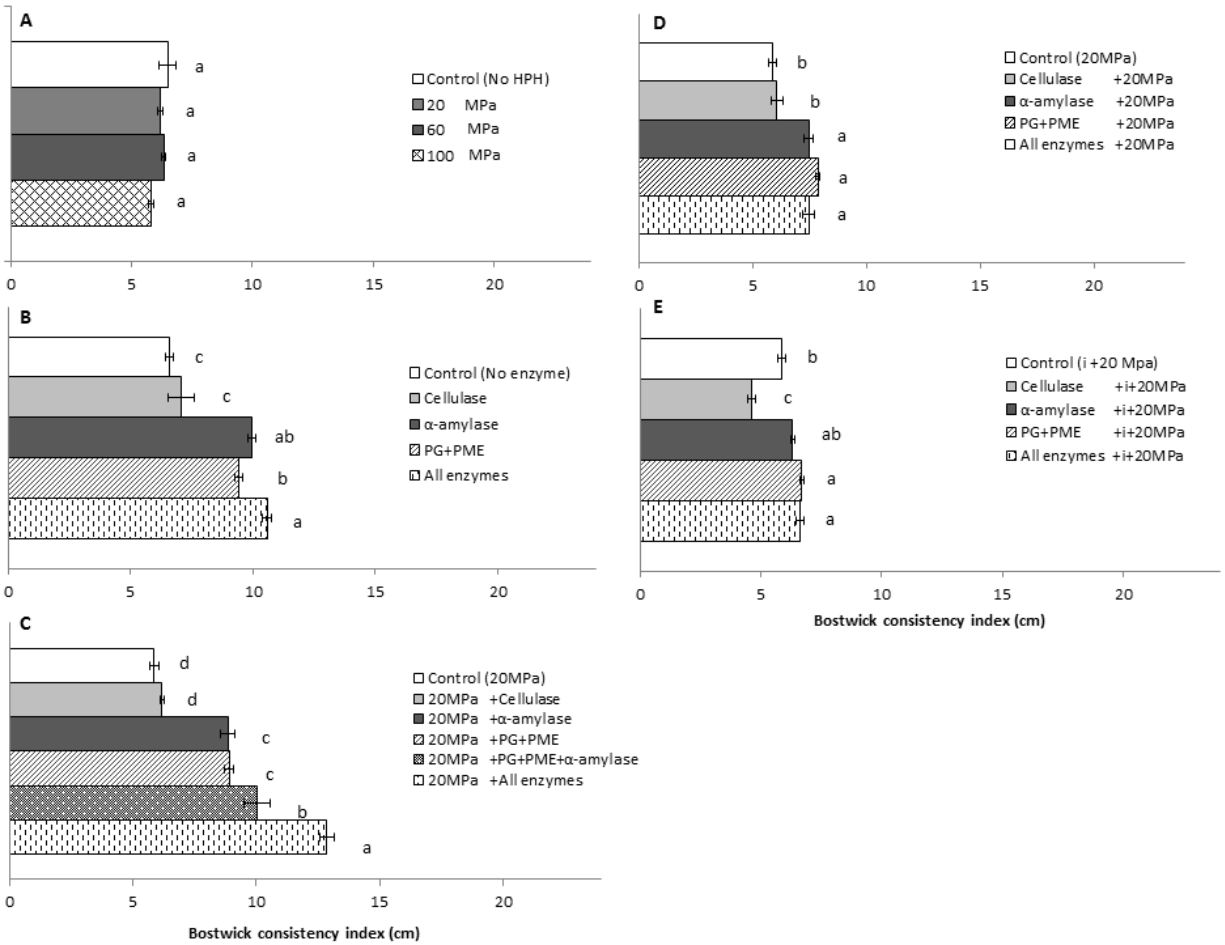


Fig. 3 Bostwick consistency index (± standard error, n=4) of differently mechanical and enzymatic treated purée. (A) mechanical treatment (HPH: high pressure homogenisation), (B) Enzymatic treatment, (C) HPH followed by enzymatic treatment, (D) Enzymatic treatment immediately followed by HPH, (E) incubation of purée with enzymes followed by HPH. "i" stands for incubation.

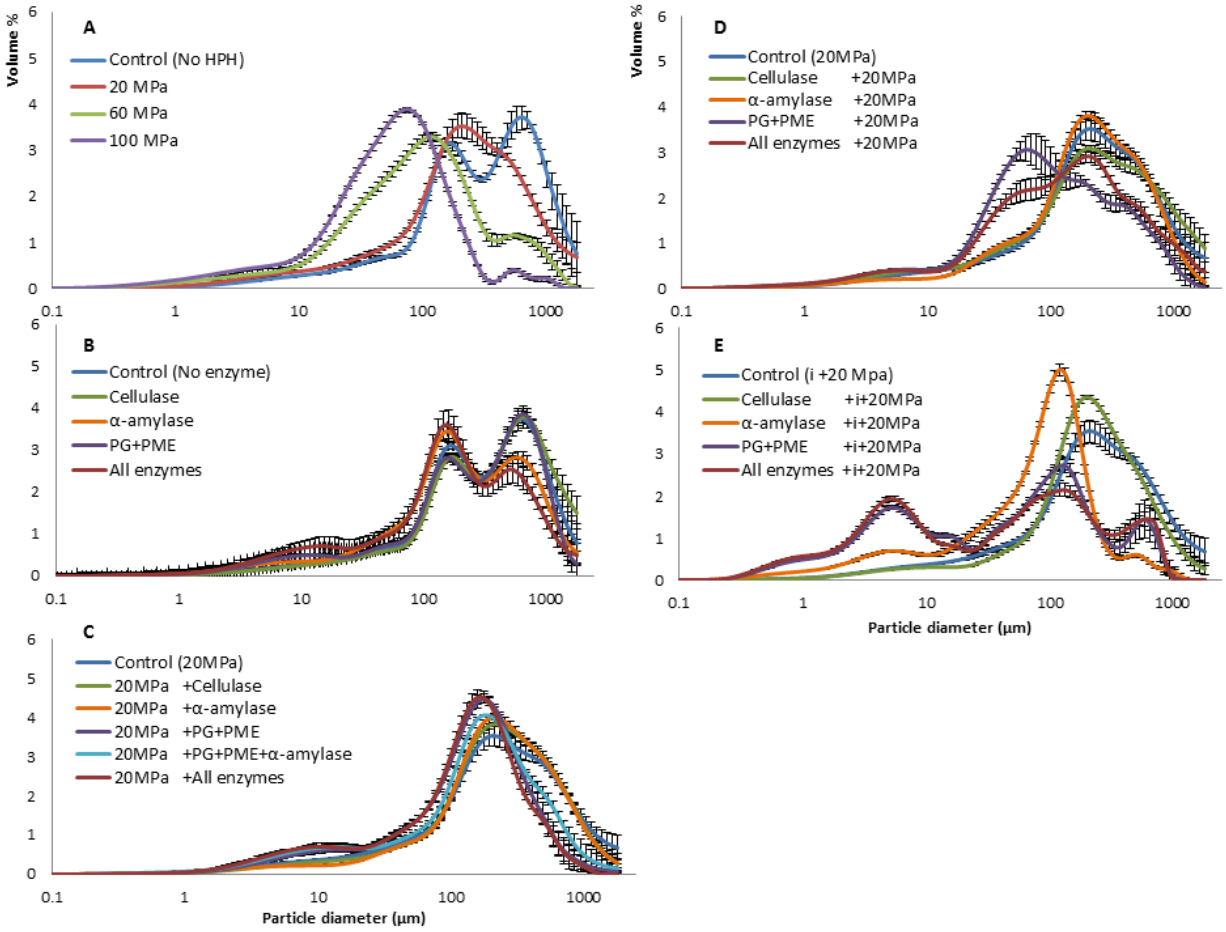


Fig. 4 Volumetric particle size distribution (± standard error, n=4) of differently mechanical and enzymatic treated purées. (A) mechanical treatment (HPH: high pressure homogenisation), (B) Enzymatic treatment, (C) HPH followed by enzymatic treatment, (D) Enzymatic treatment immediately followed by HPH, (E) incubation of purée with enzymes followed by HPH. For interpretation of the references to colors in this figure legend, the reader is referred to the web version of this article.

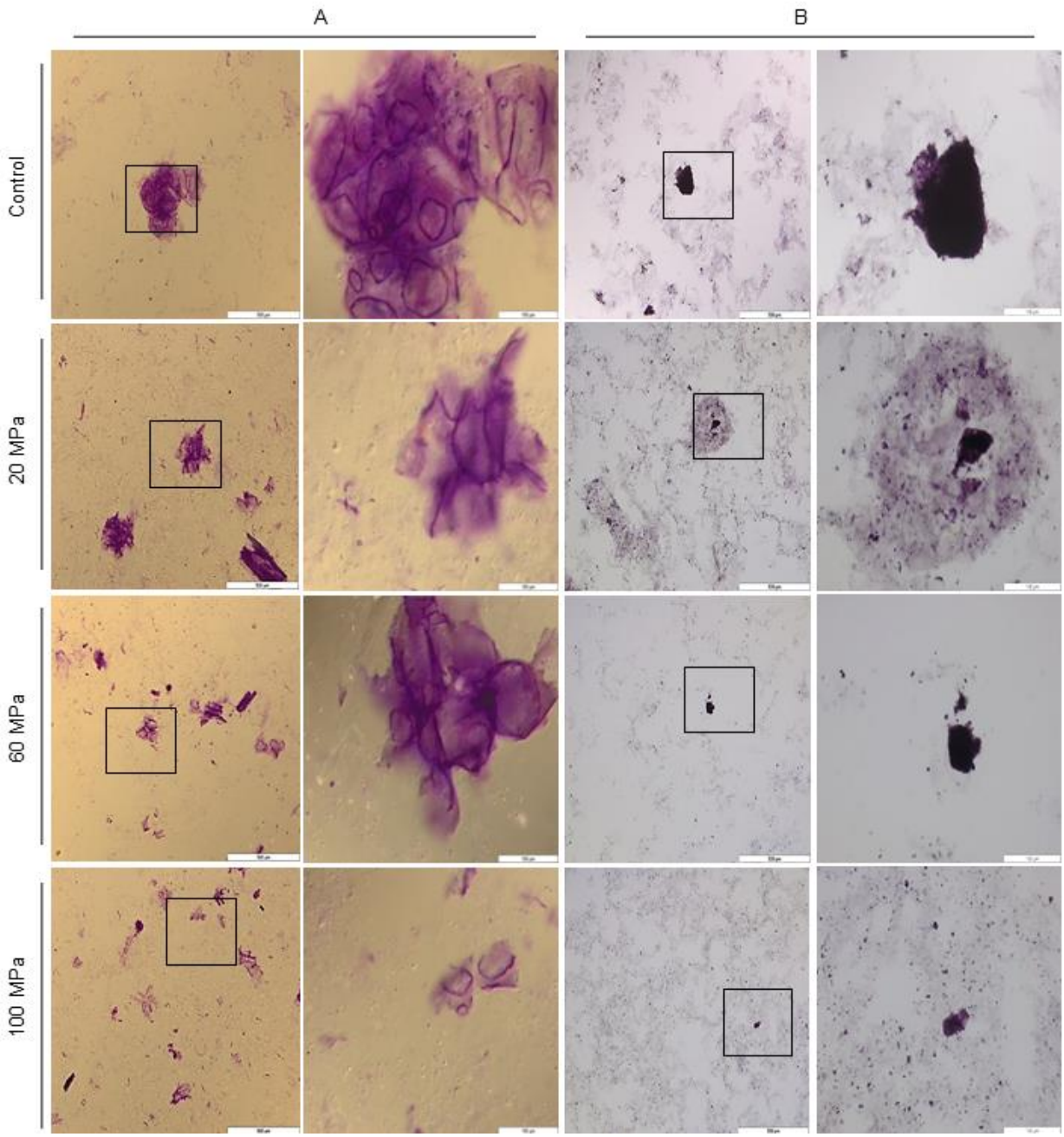
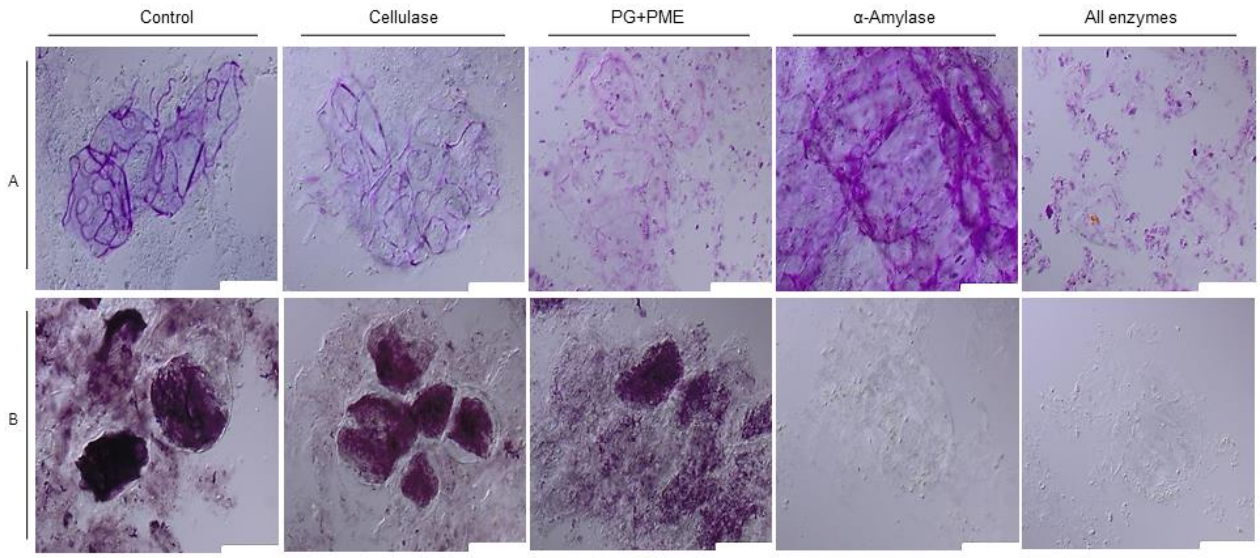


Fig. 5 Microscopic images of high pressure homogenised purée particles stained by (A) toluidine blue and (B) iodine solution. The scale bars for the first and the third column from the left are 500 µm and the second and the forth from the left are 100 µm.



635

636 **Fig. 6** Microscopic images of enzymatic treated purée particles stained by (A) toluidine blue and (B) iodine
637 solution. Scale bars = 100 μm.

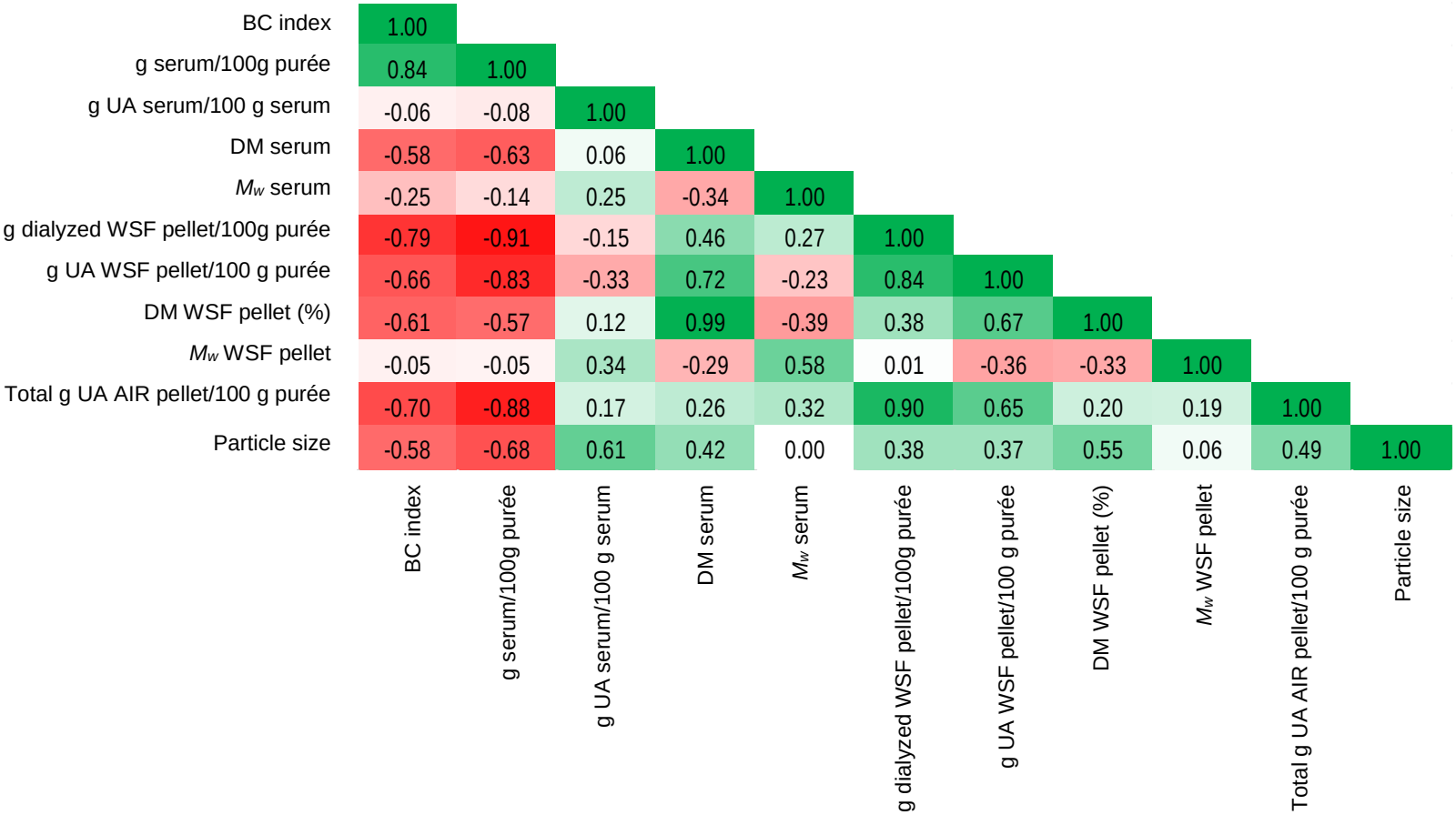


Fig 7. Correlation plot between Bostwick consistency index and parameters influenced by the ezymatic treatment and the combination of HPH and enzymatic treatment. BC index; Bostwick consistency index, UA; uronic acid, DM; degree of methoxylation, M_w; average molecular weight, WSF; water solubilised fraction, AIR; alcohol insoluble residue. The values for the particle size were the particle diameter at which 90 vol.% of the particles have a smaller diameter.

Table 1. Enzyme activity and incubation pH for the exogenous enzymes applied.

Enzyme	Activity	Incubation pH
Cellulase	1200 U/ml	4.5
PG	5000 U/ml	5.5
PME	35 U/ml	
α-amylase	2135 U/g	6.0

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